RISK ASSESSMENT OF GENE TRANSFER FROM TRANSGENIC RAPESEED TO WILD SPECIES IN OPTIMAL CONDITIONS.

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INTRODUCTION

Genetic transformation is now routinely applied to improve agronomical characters of different cultivated species. So a risk assessment is required before cultivation of transgenic plants by farmers. The first step in such a project is to estimate gene dispersal by pollen transport within the species and by outcrossing to related weed species. The first experiments are currently done in an EEC - BAP poject : a transgenic rapeseed produced by PGS (Belgium), resistant to a herbicide (Basta) .

We know that it is possible to obtain interspecific hybrids between rapeseed and different *Brassicaceae* (Harberd and Mc Arthur, 1980, Prakash and Hinata, 1980) . Two aspects of gene dispersal by crossing to wild species were studied : production of interspecific hybrids either by spontaneous pollination under field conditions or by manual pollination in greenhouse. The results obtained with this last approach will be presented in this report. The most common wild species in Europe were chosen.

MATERIAL AND METHODS

PLANT MATERIAL :

A canadian Spring rapeseed variety (Westar) was transformed using *Agrobacterium tumefaciens*. The **bar** gene introduced confers resistance to the herbicide phosphonitricin (commercial name : Basta^R). The technique of the transformation was described by De Block *et al* (1987).Progeny of different individual transformed plants with different copy number was used. Different weeds and cultivated *Brassicaceae* were used for the crosses:

- *Brassica oleracea* L. (CC , n = 9) , a wild population of var. *acephala* provided by G. THOMAS (INRA , Rennes) and a pure line of var. *capitata* provided by C. DORE (INRA , Versailles)
- *Brassica nigra* (L.) Koch (BB , n = 8) , a german variety, Junius
- *Brassica adpressa* L. (Ad Ad , n = 7)
- *Raphanus raphanistrum* L. (RR , n = 9)
- *Sinapis arvensis* L. (Sar Sar , n =9)

For the three last species, population were collected locally.

OVARY CULTURE :

Reciprocal crosses were produced. The buds were emasculated and pollinated. Four to six days after pollination, ovaries were excised and established in *in vitro* culture. The technique and the composition of E12 medium was given by Delourme *et al* (1989).The cultures were incubated in a growth chamber at 22-23° day/15° night with a 16 h photoperiod. When they appeared, embryos were placed during 10 days at 4° C and then transferred on Murashige and Skoog (1962) medium, containing 20 g/l of sucrose. At the 4 to 6 leaf stage, plantlets were transferred in pots in the greenhouse.

CHROMOSOME COUNTING :

It was performed in root-tip dividing cells or in pollen mother cells. The observation techniques were previously described by Jahier *et al* (1987) and Chèvre *et al* (1991) .

POLLEN FERTILITY :

It was estimated by determining the percentage of pollen grains stained by aceto - carmine. About 500 grains were observed per plant.

GENE CHARACTERIZATION :

The presence of the **bar** gene was detected by Polymerase Chain Reaction (PCR) . Two primers were used to amplify a 424 bp fragment of the **bar** gene. Five µl of an extracted DNA according the simplied Dellaporta technique (1983) was used in the amplification reaction. DNA was previously denatured during 10 minutes at 95°C . PCR reaction was performed in volumes of 80 µl containing 10 mM Tris - HCl, pH = 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % of bovine albumin (BSA) , 0.2 µM of dNTPs, 10 µM primers, genomic DNA and 2 U of Taq DNA polymerase. Thermal cycler was programmed for 35 cycles of 1 min at 95°C, 1 min at 47°C and 2 min at 72°C . Amplification products were analyzed by electrophoresis in 1.5 % agarose gels containing 0.03 % of ethidium bromide.

The expression of the **bar** gene in the hybrids were studied by spraying a 1 % Basta solution containing 0.1 % on young leaves. Five young leaves per plants were sprayed. Observations were made 7 to 10 days after treatment.

RESULTS

From reciprocal crosses between the different *Brassicaceae* species and the transgenic rapeseed, 109 hybrids were obtained (Table 1) . No seed was produced without embryo rescue.

A maternal effect was demonstrated : *B. napus* as female parent gave better results except with *R. raphanistrum* for which no difference was detected. Hybridizations with *B. nigra* or *S. arvensis* were unsuccessful when they were used as female.

Crosses between *B. napus* as female and *B. oleracea* var *acephala* gave better results, with 13.65 hybrids per 100 ovaries. For the other hybridizations using *B. napus* as female, no signifiant difference was found by X^2 test.

The percentage of hybrid plants from crosses using *B. napus* as male allowed us to define 2 groups by X^2 test : one with *R. raphanistrum*, *B. adpressa* and *B. oleracea* var *acephala* and the other one with var. *capitata*, *B. nigra* and *S. arvensis*.

Chromosome counting in root meristem cells or pollen mother cells demonstrated that most of the hybrids had the expected triploid structure (ACX) : one genome rapeseed (AC) and one genome of the species used (X). Hybrids had 26 or 27 chromosomes from crosses with *B. adpressa*, *B. nigra* respectively and 28 chromosomes from crosses with *B. oleracea*, *R. raphanistrum* and *S. arvensis*. Three amphidiploids with 56 chromosomes (AACCXX) were obtained : 2 from hybridizations with *B. oleracea* and one from hybridizations with *S. arvensis*.

Pollen fertility is reported in Table 1. Hybrids obtained from *B. adpressa* were sterile whatever the female parent, whereas hybrids with *R. raphanistrum* and *B. nigra* were either sterile or partially fertile (never more than 7.1 %) . In the same way , different hybrids produced with *S. arvensis* had pollen fertility ranging from 0 to 34 % . For *B. napus* - *B. oleracea* hybrids, sterile and fertile florets were present on the same plant except for one sterile hybrid. From the fertile flowers, the pollen fertility was 20.7 % (7.9 to 59.2 %) and 13.5 % (4.5 to 40.2 %) from var *acephala* and var. *capitata* respectively. The amphidiploids *B. napus* - *B* *oleracea* were as fertile as *B. napus* parent (94 %) whereas *B. napus - S. arvensis* amphidiploid had the same fertility as the F1 hybrids (34.8 %) .

The **bar** gene was characterized by studying its presence (PCR) and its expression (Basta treatment) (Table 2) . Only hybrids obtained from transgenic rapeseed with 1 or 3 copies of the **bar** gene were analyzed. From the preliminary results, a good correlation was established between the presence of **bar** gene and the Basta resistance except for two plants. All the susceptible hybrids without PCR amplification product were obtained from 3 copy transgenic rapeseed.

DISCUSSION

Hybrids between *B. napus* and *B. oleracea, B. adpressa, B. nigra, S.arvensis* were previously described by different authors (Mizushima 1950 a, Heyn 1977, Harberd and McArthur 1980, Busso *et al* 1987, Inomata 1988, Quazi 1988, Jahier *et al* 1989) . The only hybrids between *B. napus* and the genus *Raphanus* were obtained from *R. sativus* (Eber, com. pers.) . For the first time, reciprocal hybrids between *B. napus* and *R. raphanistrum* were produced. Although hybrids between *B. napus - B. adpressa* by using this last species as male, were previously described (Harberd and McArthur, 1980) , we reporte in the present study the characterization of these hybrids to the two cytoplasms.

Hybridizations were most successful when the rapeseed was used as female except for the crosses with *R. raphanistrum*. These results are in agreement with previous observations which reported that crosses may be more successful if the parent with the highest chromosome number is used as female parent (Johnston 1980, Mohapatra and Bajaj, 1987, Quazi, 1988) .

Different interspecific incompatibility systems with *Brassicaceae* species have been described (Meng and Liu, 1987) . This could explain the difficulties we had to produce hybrids between *B. nigra - B. napus* and *S. arvensis - B. napus* . However a varietal effect can be taken into account. Busso *et al* (1987) produced hybrids with *B. nigra* as female parent, and Ripley and Arnison (1990) demonstrated a varietal effect for the creation of *S. alba - B. napus* hybrids. This observation was confirmed by the difference we found between the two varieties of *B. oleracea* used, var. *acephala* and var. *capitata* for hybrid production.

Most of the hybrids had the expected triploid structure (ACX) except 3 amphidiploids with 56 chromosomes. This chromosome doubling could be explained by endomitosis during the *in vitro* culture. This probability is very low since embryos were well formed before subculture and it seems difficult that somaclonal variation occurred. The presence of efficient non reduced gametes in the parental plants was more probable. Mizushima (1950 b) reported that two amphidiploids could be obtained after crosses between *B. napus* and *S. arvensis*. The same hypothesis was proposed by Heyn (1977) to explain the results obtained with crosses between *B. napus* and *Eruca sativa*.

The F1 hybrids were either sterile or poorly fertile. The cytoplasm did not seem to affect the fertility percentage which was similar independently of the origin of the mother - plant for hybrids *B. napus - B. adpressa* or *B. napus - B. oleracea*. The diploid structure and the regular meiotic behavior (data not shown) could probably explain the good fertility of the amphidiploids *B. oleracea - B. napus* obtained. However, while Mizushima (1950 b) reported that amphidiploid *B. napus - S. arvensis* had 80 % fertility, the plant we obtained was as fertile as F1 hybrids.

As we expected the presence of the **bar** gene was correlated with the Basta resistance. However the **bar** gene was also found in two susceptible plants. As we used for producing these plants a 3 copy transgenic rapeseed, it is possible that one of the copy transmitted to these hybrids had an initial insertion site which did not allow the expression of the gene. After crosses the insertion site could be also modified; Morota and

Uchimiya (1988) correlated a non expression of the nopaline synthetase in the progeny of a transgenic tabacco to an alteration of the **nos** gene. The hypothesis of a regulation of the inserted gene by the genomes present in an interspecific hybrid could be also proposed.

CONCLUSION

A precise characterization of the insertion sites of the initial transgenic rapeseed plants (especially the 3 copy one) will be performed to explain the difference observed between hybrids containing the **bar** gene.

We showed that it was possible to perform the first step in the gene transfer to wild species : production of interspecific hybrids. In a second step, it will be necessary to assess the capability of these hybrids to produce progeny after backcrossing with the wild species. This study is underway. Seed set seems more efficient from amphidiploid than from F1 hybrids (data not shown) .

In the backcrossing progeny , the study of the presence and of the expression of the **bar** gene and of different markers will allow us to assess the probability of recombination between rapeseed and wild species genomes.

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TABLE 1 : PRODUCTION AND CHARACTERIZATION OF HYBRIDS BETWEEN TRANSGENIC RAPESEED AND WILD SPECIES

Interspecific crosses		Hybrid Production			Cytogenetic characterization		
wild species	transgenic B.napus	Number of ovaries in culture A	Number of plantlets obtained B	B/A*100	Number of plants studied	2n	Pollen fertility %
B.oleracea V.acephala							
M	F	205	28	13.65	27 1	28 56	7.6-59.2 94.1
F	M	445	3	0.41	3	28	1.2-18.8
B.oleracea V.capitata							
M	F	228	13	7.70	9 1	28 56	4.5-40.0 94.0
F	M	585	1	0.17	1	28	16.6
B.nigra							
M	F	325	11	3.38	6	27	0.0-1.9
F	M	916	0	0	0		
B.adpressa							
M	F	262	8	3.05	6	26	0.0
F	M	1117	15	1.34	10	26	0.0
S.arvensis							
M	F	808	18	2.23	11 1	28 56	0.0-39.8 34.8
F	M	732	0	0	0		
R.raphanistrum							
M	F	368	3	0.81	1	28	*
F	M	583	9	1.54	8	28	0.0-7.1

M : Male F : Femelle * : studies in progress

TABLE 2 : CHARACTERIZATION OF THE BAR GENE IN THE HYBRIDS

Interspecific crosses		Number	Origin of the transgenic rapeseed: one copy of the Bar gene			3 copies of the Bar gene		
wild species	transgenic B.napus	of plants studied	PCR+ Basta R	PCR+ Basta S	PCR- Basta S	PCR+ Basta R	PCR+ Basta S	PCR- Basta S
B.oleracea V.acephala								
M	F	11	-	-	-	6	0	5
F	M	2	-	-	-	2	0	1
B.oleracea V.capitata								
M	F	8	3	-	-	3	-	2
F	M	1	-	-	-	1	-	-
B.nigra								
M	F	6	3	-	-	1	1	-
F	M	0	-	-	-	-	-	-
B.adpressa								
M	F	0	-	-	-	-	-	-
F	M	5	4	-	-	1	1	-
S.arvensis								
M	F	1	1	-	-	-	-	-
F	M	-	-	-	-	-	-	-
R.raphanistrum								
M	F	1	1	-	-	-	-	-
F	M	2	2	-	-	-	-	-